Early Development and Acquisition of Zooxanthellae in the Temperate Symbiotic Sea Anemone *Anthopleura ballii* (Cocks)

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Abstract. The ova of *Anthopleura ballii* become infected with zooxanthellae (endosymbiotic dinoflagellates) of maternal origin just prior to spawning. After fertilization, the zygotes undergo radial, holoblastic cleavage, and then gastrulate by invagination to form ciliated planulae. Because the zooxanthellae are localized on one side of the ovum—and later, within the blastomeres at one end of the embryo—invagination leads to the zooxanthellae being restricted to the planular endoderm and hence to the gastrodermal cells of the adult anemone. We propose that maternal inheritance of zooxanthellae plays an important part in the success of these temperate sea anemones, which live in regions where potential sources of zooxanthellae are scarce.

Introduction

Associations between marine invertebrates and endosymbiotic dinoflagellates (zooxanthellae) are abundant in nutrient-poor tropical seas, where the zooxanthellae supply photosynthetically fixed carbon to their hosts and facilitate the conservation and recycling of essential nutrients (Muscatine, 1990; Davies, 1992). These nutritional advantages are not immediately obvious in temperate waters, which show marked seasonal fluctuations in irradiance, high levels of nutrients, and seasonal abundance of planktonic food (Davy *et al.*, 1996, 1997; Muller-Parker and Davy, 2001). Indeed, associations between invertebrates and zooxanthellae are uncommon at temperate latitudes (Turner, 1988; Davy *et al.*, 1996; Muller-Parker and Davy, 2001).

Hosts may acquire zooxanthellae either by maternal inheritance or from the surrounding seawater. Maternal inheritance is probably the rarer mechanism in the tropics. For example, while some reef corals inherit their symbionts (Lewis, 1974; Kojis and Quinn, 1981; Richmond, 1981; Babcock *et al.*, 1986; Glynn *et al.*, 1991), the vast majority of coral species spawn gametes that lack zooxanthellae (Babcock *et al.*, 1986).

In contrast to tropical symbioses, for temperate symbioses transmission modes have been identified in only a few cases. These include the soft coral *Capnella gaboensis*, which inherits zooxanthellae from the parent colony (Farrant, 1986); the scleractinian coral *Astrangia danae*, which spawns zooxanthella-free gametes (Szmant-Froelich *et al.*, 1980); and a small number of sea anemones, the majority of whose ova contain algal symbionts (reviewed by Shick, 1991; Muller-Parker and Davy, 2001). Moreover, the cellular events leading to the acquisition of zooxanthellae and their eventual restriction to the host's endodermal cells have been reported for tropical scleractinian corals (Hirose *et al.*, 2000, 2001), soft corals (Benayahu *et al.*, 1988, 1992; Benayahu and Schleyer, 1998), and jellyfish (Montgomery and Kremer, 1995), but not for temperate corals or sea anemones.

The sea anemone *Anthopleura ballii* (Cocks) is locally abundant along the southwestern coasts of Europe, where it is found from intertidal regions to depths of about 25 m (Manuel, 1988; Turner, 1988; Davy *et al.*, 1996, 1997). The zooxanthellae harbored by *A. ballii* belong to the genus *Symbiodinium* (Davy *et al.*, 1997), though they have yet to be subjected to molecular characterization. In this study, we documented cellular events from gametogenesis through to planula development in *A. ballii*, paying particular attention

to the transmission and distribution of zooxanthellae within the host's tissues.

Materials and Methods

Experimental organisms

Specimens of the zooxanthellate sea anemone *Anthopleura ballii* were collected, from between 0 and 25 m depth, in the Lough Hyne Marine Nature Reserve, Eire. Anemones were then maintained for up to one year in 30-l recirculating seawater tanks at 10–15 °C. Irradiance of 96 μmol photons m^{-2} s^{-1} was provided on a 12-h light:12-h dark regime, and the anemones were fed twice weekly with *Artemia* sp. nauplii.

Microscopical examination

Spawning of *A. ballii*, which is dioecious, was induced during summer. This was done by exposing anemones to air for between 3 and 5 h. The expelled gametes were collected by pipette and maintained in 100-ml sterile flasks containing artificial seawater at 15 °C. Fertilization occurred within hours and, every second day, the embryos were pipetted into new flasks, which also contained artificial seawater. This procedure ensured that the only possible source of zooxanthellae was the adult anemone.

Gametes, fertilization, and subsequent early development were examined by taking samples, first at hourly intervals and later once daily, for microscopical observation. A careful search for zooxanthellae was made, using interference contrast microscopy, by optical sectioning at each developmental stage. A Leitz Dialux 20 microscope with Vario-orthomat photographic system was employed, and a photographic record of early development was produced. In addition, cellular events occurring during gametogenesis were documented, again using interference contrast microscopy. This was made possible by anesthetizing anemones in equal parts artificial seawater and 7.5% $MgCl_2 \cdot 6H_2O$ for 12 to 24 h, and then teasing gametes out of the gonads.

Results

Gametogenesis and gametes

Dissection occasionally revealed germ cells in the gonadal tissue on the mesentery. The mesenterial tissue was densely packed with zooxanthellae, and the tissues around the oocytes contained many zooxanthellae, but the oocytes themselves were never observed to contain zooxanthellae (Fig. 1A).

Unfertilized ova, examined by interference phase microscopy immediately upon release from the adult anemone, were spherical, yellow-brown, and 300 μm in diameter (Figs. 1B, 2A). The surface of each ovum was covered in fine translucent cytospines (stiffened bundles of long mac-

rovilli that are characteristic of actiniarians; Larkman, 1980), about 23 μm in length. The cytoplasm was heterogeneous, dense and granular, and no nuclei were visible under low power in unstained preparations. When the ova were optically sectioned by interference contrast microscopy, the zooxanthellae could be observed in the cytoplasm, just inside the cell membrane. Moreover, the zooxanthellae were concentrated in one hemisphere of the ovum (Fig. 1B). Out of a total of 380 ova examined, only one aposymbiotic ovum was observed.

Spermatozoa were examined under high power ($\times$ 1000) in a live preparation. The head was rectangular, 3.5 μm in length and 2.3 μm across, with dense cytoplasm and a large, dark nucleus. No basal body was visible. The tail was about 50 μm long.

Fertilization

The gametes were shed into open water, where fertilization occurred. Each released ovum was surrounded by numerous sperm, which were aggregated between the cytospines. Fertilization usually occurred within 3.5 h of spawning, and unfertilized ova disintegrated after about 7 h, liberating their zooxanthellae. Spermatozoa were still active at this stage and became inactive after 20–32 h.

Cleavage

About 3.5 h after spawning, 2-, 4-, 8-, and 16-cell blastulas were observed (4- to 16-cell stages shown in Fig. 1C-1E; 2-cell stage not shown). Cleavage was equal, radial, complete (*i.e.*, holoblastic), and rapid, dividing the embryo into a ball of cells (blastomeres). Due to the initial localization of the zooxanthellae, symbionts were distributed unevenly in the blastomeres, being concentrated in those cells at only one end of the embryo. The zooxanthellae remained just inside the cell membrane of each blastomere. Blastulas of 32 cells were observed after about 5.5 h, and blastulas of 64 cells or more were apparent after 6 h. The blastomeres became ever smaller due to repeated cleavage, and after 8 h, a coeloblastula consisting of many cells and one cell layer was formed (Figs. 1F, 2B). The blastomeres were now 20–30 μm in diameter, even in size, and rarely contained more than one zooxanthella each (Fig. 1G). The cytospines were resorbed and replaced by cilia, which soon began to exhibit the characteristic metachronal rhythm that rendered the coeloblastula motile.

Gastrulation

Few gastrulae (Figs. 1H-I, 2C-E) were seen, suggesting that this developmental stage is very short. Twenty hours after spawning, the motile coeloblastulas began to show a slight depression at the pole about which the algae were
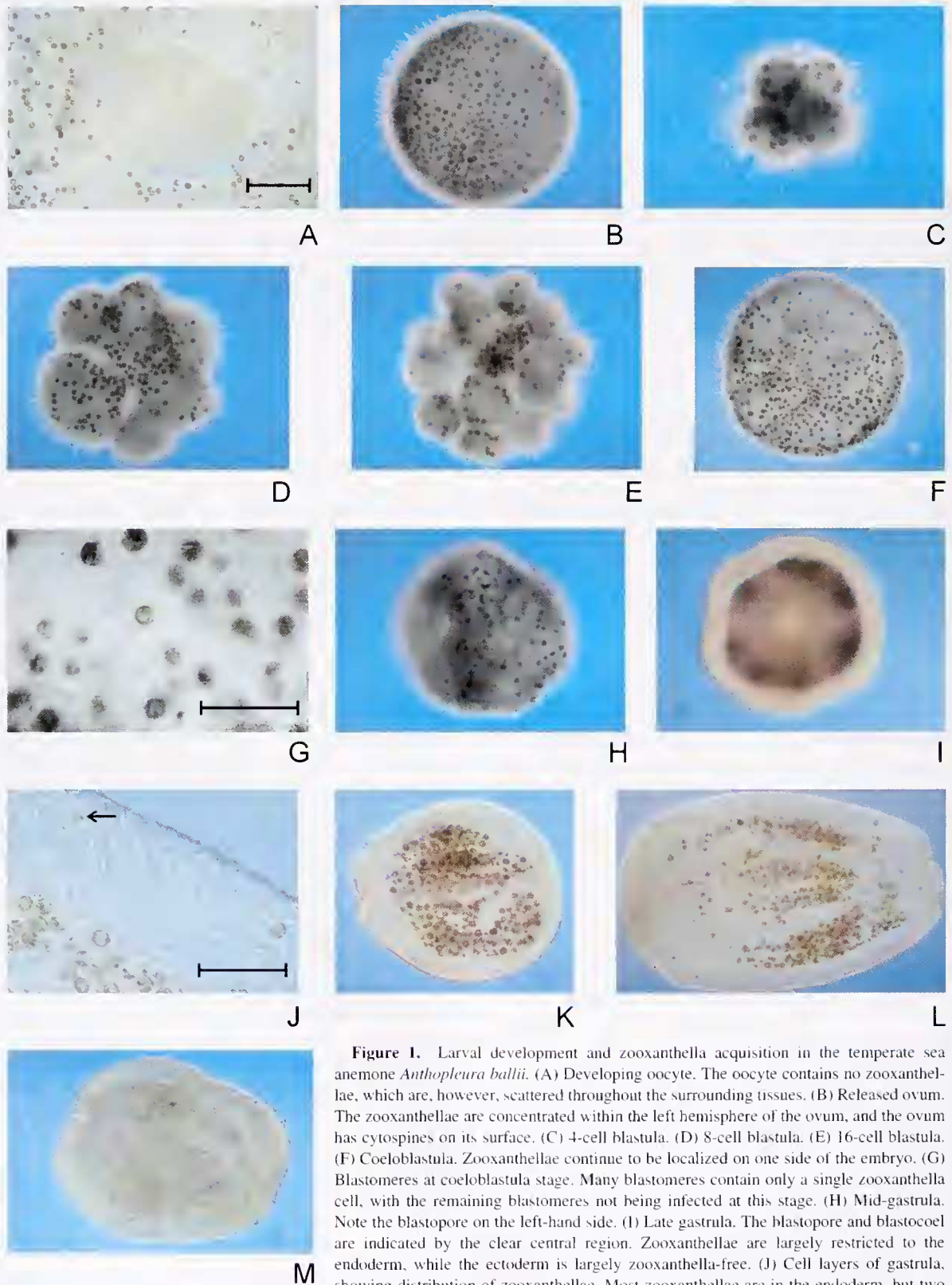
Figure 1. Larval development and zooxanthella acquisition in the temperate sea anemone *Anthopleura ballii*. (A) Developing oocyte. The oocyte contains no zooxanthellae, which are, however, scattered throughout the surrounding tissues. (B) Released ovum. The zooxanthellae are concentrated within the left hemisphere of the ovum, and the ovum has cytospines on its surface. (C) 4-cell blastula. (D) 8-cell blastula. (E) 16-cell blastula. (F) Coeloblastula. Zooxanthellae continue to be localized on one side of the embryo. (G) Blastomeres at coeloblastula stage. Many blastomeres contain only a single zooxanthella cell, with the remaining blastomeres not being infected at this stage. (H) Mid-gastrula. Note the blastopore on the left-hand side. (I) Late gastrula. The blastopore and blastocoel are indicated by the clear central region. Zooxanthellae are largely restricted to the endoderm, while the ectoderm is largely zooxanthella-free. (J) Cell layers of gastrula, showing distribution of zooxanthellae. Most zooxanthellae are in the endoderm, but two can be seen in the ectoderm. One of these zooxanthellae (see arrow) appears to be degenerating. (K) Early planula. (L) Late planula. Zooxanthellae are distributed along the mesenteries. (M) Aposymbiotic mid-planula. Scale bar in A applies to all images except G and J and represents about 100 µm; scale bars for G and J represent about 50 µm.
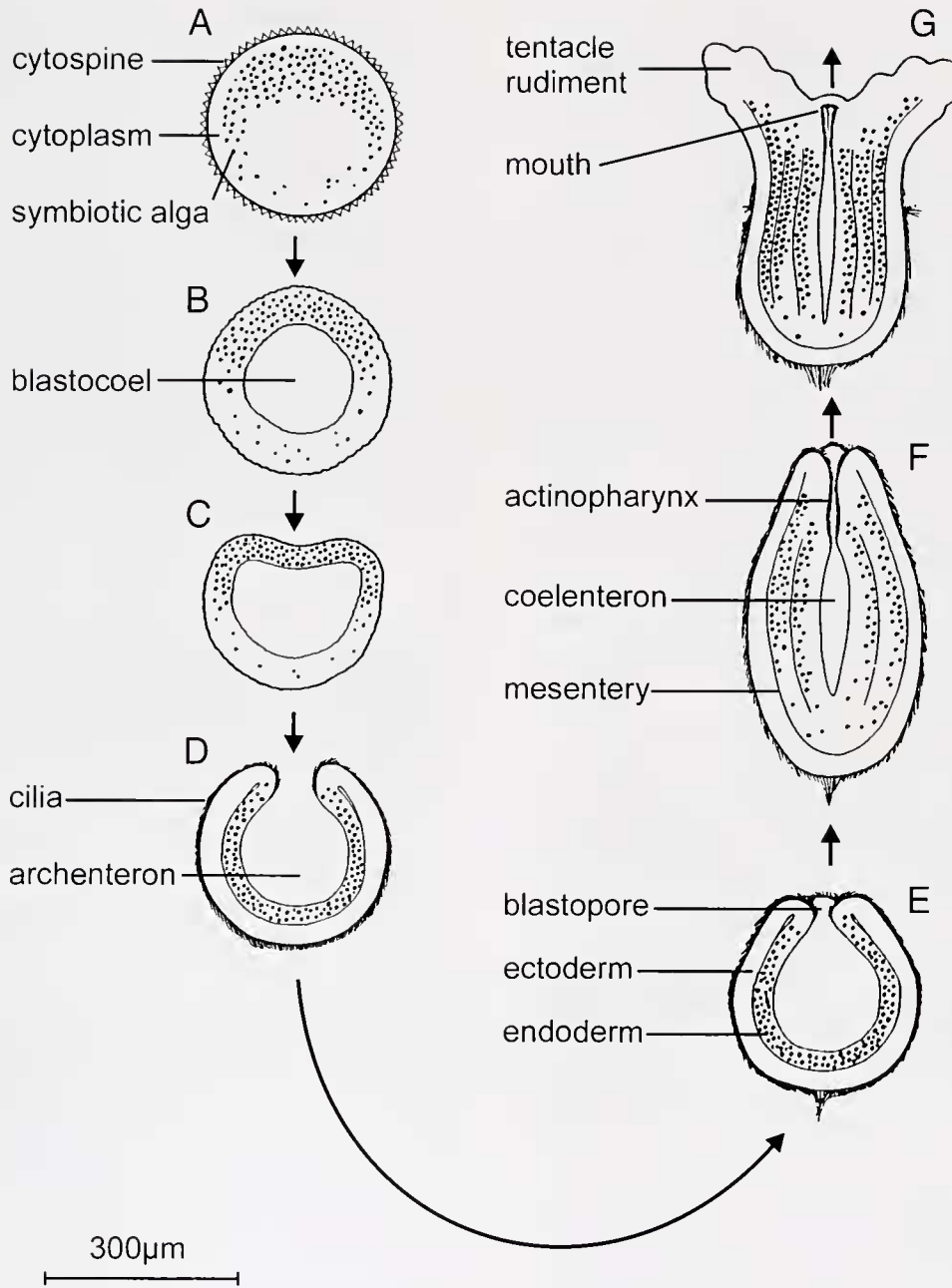
Figure 2. Localization of zooxanthellae during early development of *Anthopleura ballii*. (A) Ovum, showing localization of zooxanthellae. (B) Coeloblastula. (C) Early gastrula, showing invagination. (D) Midgastrula, showing localization of zooxanthellae in the endoderm. (E) Late gastrula-early planula. (F) Midplanula, showing zooxanthellae distributed along the mesenteries. (G) Late planula, prior to settlement.

concentrated. Gastrulation by invagination (and perhaps epiboly) followed (Figs. 1H, 2C), with the blastomeres aggregated, at first, around the blastopore. Gastrulation led to the formation of an embryo with two cell layers encompassing a central cavity—the archenteron (Figs. 1I, 2D). During gastrulation, almost all of the blastomeres containing zooxanthellae moved into the endoderm from around the blastopore region. Only very occasionally were zooxan-

thellae seen in the ectoderm, and many of these cells appeared to disintegrate (Fig. 1J).

Planulation

After 27 h, most embryos had become late gastrulae or early planulae (Figs. 1K, 2E). By this stage, the developing larvae had shown no growth, remaining about 300 μm in

diameter. However, after 2 days, most planulae began to elongate along their vertical axis, tapering slightly towards the posterior end. The zooxanthellae were clearly visible, aggregated in striations running the length of the endoderm. The surface of each larva was completely ciliated, and an apical tuft of longer cilia was visible. After 3 days, the larvae began to exhibit signs of differentiation (Figs. 1L, 2F), with the development of nematocysts, and a ciliated actinopharynx, which replaced the blastopore. Between 3 and 5 days, the planulae began to grow to about 400–600 μm in length and 300 μm in diameter, even though they were not fed. The number of zooxanthellae also increased (not quantified), and dividing zooxanthellae were seen frequently. As the mesenteries developed, it became clear that most zooxanthellae were located along these structures. Interestingly, only one aposymbiotic planula was observed throughout the course of this work (Fig. 1M), which is consistent with the absence of aposymbiotic A. ballii at the field site (Lough Hyne). Tentacle rudiments were seen very occasionally in some planulae (Fig. 2G). Although care was taken to isolate the surviving planulae, they could not be kept alive for more than 7 days and so settlement was not observed.

Discussion

Gametogenesis, spawning, and early development in *Anthopleura ballii* follows the pattern exemplified by many anemone species (Siebert, 1973; Chia, 1976; Jennison, 1979, 1981). All these species are dioecious, shedding their gametes into open water where fertilization occurs. The zygote then undergoes radial, holoblastic cleavage and forms a hollow coeloblastula. Gastrulation follows by invagination to form a ciliated, pelagic planula larva. This mode of development is notably different from that shown by the larger, yolky, meroblastic ova of the anemones *Tealia crassicornis* (Chia and Spaulding, 1972) and *Cribrinopsis fernaldi* (Siebert and Spaulding, 1976), in which cleavage is incomplete, unequal, and relatively slow. The sequence and timing of events in *A. ballii* were very similar to those described for the temperate zooxanthellate or zoochlorellate anemones *Anthopleura elegantissima* and *Anthopleura xanthogrammica* (Siebert, 1973). However, unlike these anemones, *A. ballii* spawned ova that contained zooxanthellae. In *A. ballii*, concentration of the zooxanthellae in one hemisphere of the ovum, and invagination (and perhaps epiboly) during the gastrula stage, led to the localization of symbionts within the host's endoderm; this same process occurs in the temperate anemone *Anemonia viridis* (Turner, 1988).

Gametogenesis and zooxanthella acquisition

In *A. ballii*, the endodermal tissue surrounding the developing oocytes was heavily laden with zooxanthellae, though infected oocytes were never observed. In contrast, spawned ova almost always harbored zooxanthellae, indicating that infection must occur at, or just prior to, release. We could not ascertain whether infection occurs in the gonadal tissue or after the ova have been released into the coelenteron. But, as the anemones were kept in artificial (and so zooxanthella-free) seawater in sterile flasks, and as spawning occurred in air, we can be certain that the zooxanthellae were of maternal origin, and that infection occurs prior to release into the surrounding seawater and hence prior to fertilization.

While the mode of zooxanthella acquisition has been determined in relatively few species of cnidarians, early indications are that infection prior to fertilization is quite uncommon. For example, the vast majority of scleractinian corals investigated do not harbor zooxanthellae in their eggs (Szmant-Froelich *et al.*, 1980; Babcock *et al.*, 1986; Harrison and Wallace, 1990), though some species of *Pocillopora* and *Montipora* do release zooxanthellate ova (Babcock *et al.*, 1986; Harrison and Wallace, 1990; Glynn *et al.*, 1991; Hirose *et al.*, 2001). Of note, the eggs of the hard coral *Montipora digitata* become infected just 24 h prior to spawning (Harrison and Wallace, 1990), suggesting that the delayed infection seen in *A. ballii* eggs also occurs in some other hosts. Furthermore, in brooding species like the soft corals *Xenia umbellata* and *Anthelia glauca*, where zooxanthellae are transmitted maternally, infection does not occur until the later stages of embryogenesis or larval development (Benayahu *et al.*, 1988; Benayahu and Schleyer, 1998).

The mechanism of entry into the ovum is unknown, but may well be similar to that described for the soft coral *Litophyton arboreum* (Benayahu *et al.*, 1992). In *L. arboreum*, zooxanthellae pass through gaps in the mesogloeal covering of the oocytes, accumulate in the perioocytic zone, and ultimately bulge through the oolema and enter the mature oocyte. A similar "phagocytosis" of algal symbionts has been reported for the oocytes of several scleractinian corals (Hirose *et al.*, 2001), as well as for the freshwater *Hydra viridissima* (Campbell, 1990).

Spawning, early development, and the localization of zooxanthellae

The sperm of *A. ballii* are similar to those of other *Anthopleura* spp. (Siebert, 1973). Moreover, as in other symbiotic Anthozoa, the heads are too small (3.5×2.3 μm) to act as vectors for paternal transmission of zooxanthellae; zooxanthellae in *A. ballii* are about 10 μm in diameter (Turner, 1988; Davy *et al.*, 1996).

During the early stages of development, and throughout cleavage, the zooxanthellae remain localized at one end of the embryo. By the time a coeloblastula forms, most zooxanthellae are located in individual blastomeres, at one end of the coeloblastula. That this positioning is of paramount importance for the ultimate localization of the zooxanthellae

becomes evident during gastrulation, when zooxanthellae are situated within invaginating blastomeres and so become localized within the endoderm. Indeed, the mechanism is so successful that "stray" zooxanthellae, which end up in the ectoderm, are rare (Fig. 1J).

The initial localization of zooxanthellae seen here is similar to that seen in the corals *Pocillopora verrucosa* and *P. eydouxi* (Hirose *et al.*, 2000). However, as in some other coral species (Szmant-Froelich *et al.*, 1980, 1985), gastrulation in *P. verrucosa* and *P. eydouxi* occurs *via* delamination rather than invagination. This means that, in marked contrast to events observed in *A. ballii*, blastomeres containing zooxanthellae move into the blastocoel of developing embryos, eventually filling the space and forming a stereogastrula (Hirose *et al.*, 2000). The precise mechanism by which the zooxanthellae move into the blastocoel is unknown.

Planulation

As stated above, the position of zooxanthellae in the embryo, and the subsequent localization of zooxanthellae in the endodermis by invagination, means that "stray" zooxanthellae in the epidermal cells of planulae are very rare. A similar paucity of stray zooxanthellae was also reported for the reef corals *Pocillopora verrucosa* and *P. eydouxi* (Hirose *et al.*, 2000). However, the planulae of some scleractinian corals (Szmant-Froelich *et al.*, 1985; Schwarz *et al.*, 1999), soft corals (Farrant, 1986; Benayahu *et al.*, 1988, 1992; Benayahu and Schleyer, 1998), and jellyfish (Montgomery and Kremer, 1995) may contain zooxanthellae in their epidermal cells more frequently. In these cases, the zooxanthellae infect either the planulae or, as in the jellyfish *Linuche unguiculata*, both the embryos and planulae (Montgomery and Kremer, 1995), as opposed to the gametes. The zooxanthellae may then be transferred to the endodermal tissue *via* cell migration (Montgomery and Kremer, 1995) or trans-mesogloeal passages (Benayahu, 1997; Benayahu and Schleyer, 1998). Alternatively, stray zooxanthellae may degrade in the host or be expelled as a result of being harbored by an inappropriate cell type. Degrading zooxanthellae have been observed in planulae of the scleractinian corals *Stylophora pistillata*, *Seriatopora caliendrum*, and *Pocillopora verrucosa*, though always in the endodermis, rather than the epidermis (Titlyanov *et al.*, 1998).

Mode of transmission as a function of latitude

Symbiotic invertebrates are abundant in tropical seas and regularly release zooxanthellae into the surrounding seawater (Hoegh-Guldberg *et al.*, 1987); viable zooxanthellae are also released in the feces of numerous corallivorous predators (Muller-Parker, 1984). This may result in low selective pressure for the evolution of maternal inheritance in tropical regions, as zooxanthellae are readily available from exogenous sources to infect potential hosts (Buddemeier and Fautin, 1993; Kinzie *et al.*, 2001).

In contrast, while transmission mechanisms have been investigated in relatively few species of zooxanthellate invertebrate, initial observations (including those presented here) suggest that maternal transmission of zooxanthellae is more likely to occur in temperate regions than in the tropics (reviewed by Muller-Parker and Davy, 2001). A predominance of maternal transmission mechanisms at high latitudes would not be surprising, as it could be related to a scarcity of exogenous sources of zooxanthellae and, therefore, selection against hosts that acquire their symbionts from exogenous supplies (Muller-Parker and Davy, 2001). Indeed, a scarcity of sources of zooxanthellae could explain why the temperate coral *Astrangia danae*, which does not acquire its zooxanthellae maternally, is sometimes found devoid of these symbionts (Szmant-Froelich *et al.*, 1980). In addition, maternal transmission, combined with the ability of temperate algal-invertebrate symbioses to tolerate a wide range of environmental variables (Kevin and Hudson, 1979; Squire, 2000; Howe and Marshall, 2001), could explain the persistence of zooxanthellate organisms at high latitudes (Davy *et al.*, 1997; Muller-Parker and Davy, 2001). More analyses of zooxanthellar transmission mechanisms at different latitudes, and of the ecological advantages conveyed by symbioses in nutrient-rich temperate waters, will help resolve this matter.

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